Purification and Characterization of a 2-Oxoglutarate-linked ATP-independent Deacetoxycephalosporin C Synthase of *Streptomyces lactamdurans*

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The deacetoxycephalosporin C (DAOC) synthase (expandase) of *Streptomyces lactamdurans* was highly purified, as shown by SDS-PAGE and isoelectric focusing. The enzyme catalysed the oxidative ring expansion that converts penicillin N into DAOC. The enzyme was very unstable but could be partially stabilized in 25 mM-Tris/HCl, pH 9.0, in the presence of DTT (0.1 mM). The enzyme required 2-oxoglutarate, oxygen and Fe^{2+} , but did not need ATP, ascorbic acid, Mg^{2+} or K^{+} . The optimum temperature was between 25 and 30 °C. The DAOC synthase showed a high specificity for the penicillin substrate. Only penicillin N but not isopenicillin N, penicillin G or 6-aminopenicillanic acid served as substrates. 2-Oxoglutarate analogues were not used as substrates although 2-oxobutyrate and 3-oxoadipate inhibited the enzyme by 100% and 56% respectively. The enzyme was strongly inhibited by Cu^{2+} , Co^{2+} and Zn^{2+} . The apparent K_m values for penicillin N, 2-oxoglutarate and Fe^{2+} were 52 μM , 3 μM and 71 μM respectively. The enzyme was a monomer with a molecular mass of 27 000 Da $\pm$ 1000.

INTRODUCTION

The biosynthesis of cephalosporin C in *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*) and cephamycin C in *Streptomyces clavuligerus* proceeds through a biosynthetic pathway that includes the oxidative conversion of the five-membered intermediate penicillin N into the six-membered deacetoxycephalosporin C (ring expansion) (Fig. 1) (Kohsaka & Demain, 1976; Yoshida *et al.*, 1978; Baldwin *et al.*, 1981; Jensen *et al.*, 1982). The conversion of penicillin N into deacetoxycephalosporin C (DAOC) in these micro-organisms is catalysed by the DAOC synthase (the ring expanding enzyme or expandase), a dioxygenase requiring oxygen, 2-oxoglutarate, Fe^{2+} , ascorbic acid and ATP in *A. chrysogenum* (Hook *et al.*, 1979; Kupka *et al.*, 1983*b*). The DAOC synthase of *S. clavuligerus* does not seem to require ATP (or only to a very small extent) or ascorbic acid (Jensen *et al.*, 1982). However, the instability of these enzymes has hampered the purification studies and therefore some degree of ambiguity in the cofactor requirements still persists. In *A. chrysogenum* the DAOC synthase and the DAOC hydroxylase appear to be located in the same protein (Scheidegger *et al.*, 1984) whereas in *S. clavuligerus* they appear to be separate enzymes (Jensen *et al.*, 1985).

The ability to synthesize cephamycins is widely distributed among actinomycetes. More than 25 different species of *Streptomyces* are known to produce cephamycins (Martin, 1981). However, the enzymes involved in the biosynthesis of cephamycins in those species have not been characterized with the exception of some of the enzymes in *S. clavuligerus*. It has been assumed, but is not yet proven, that cephamycins arise in all *Streptomyces* from a pathway which involves the same type of ring expansion enzyme as found in *A. chrysogenum*.

Abbreviation: DAOC, deacetoxycephalosporin C.

Fig. 1. Ring-expanding reaction catalysed by DAOC synthase that converts penicillin N into DAOC. Note the conversion of the five-membered thiazolidine ring of penicillin into the six-membered dihydrothiazine ring of the cephalosporins.

S. lactamdurans (syn. *Nocardia lactamdurans*) is a cephamycin-producing organism (Stapley *et al.*, 1972). Biosynthesis of cephamycin C in this strain and its regulation by carbon and nitrogen catabolism have been studied: synthesis of the DAOC synthase is repressed by carbon catabolism (Cortés *et al.*, 1986) and nitrogen source regulation (Castro *et al.*, 1985).

In order to get a better understanding of the regulatory mechanisms that control the synthesis and activity of this enzyme, it is necessary to clone the corresponding gene. As a first step, we have established a procedure to purify the DAOC synthase of *S. lactamdurans*.

METHODS

Micro-organisms and growth conditions. *Streptomyces lactamdurans* JC 1843, a stable variant that produces higher levels of cephamycin, was grown at 28 °C in NYG medium in an orbital incubator (250 r.p.m.) as described by Castro *et al.* (1985).

Cell-free extracts. Cells (23 g wet wt) were collected at 72 h when they contained maximum expandase activity and suspended in 60 ml TPD buffer [25 mM-Tris/HCl, pH 7.0, 0.1 mM-dithiothreitol (DTT), 1 mM-phenylmethylsulphonyl fluoride]. Cell-free extracts were obtained by sonication of the cells in a Branson Sonifier B-12 in an ice-bath for 15 s periods with 30 s intervals for a total time of 3 min. The extracts were centrifuged at 15000 *g* for 15 min in a refrigerated (4 °C) centrifuge and then at 100000 *g*, for 120 min in an ultracentrifuge (Beckman L8-70). The supernate (S100) was used as crude extract (Castro *et al.*, 1985).

DAOC synthase assay. This was done by measuring the formation of DAOC from penicillin N at 25 °C. The reaction mixture contained in a final volume of 200 μ l: 0.2 mM-penicillin N, 0.1 mM-2-oxoglutarate, 0.25 mM-FeSO₄, 50 mM-Tris/HCl, pH 7.0, and enzyme (150 μ l of a preparation containing 5–10 mg protein ml⁻¹). The DAOC produced was determined by a microbiological assay using *E. coli* Ess 22-31 as a test strain (Cortés *et al.*, 1986). The residual substrate was removed by doing the bioassay in the presence of penicillinase (*Bacillus cereus* UL-1) lacking cephalosporinase activity. Penicillinase was mixed with TSB (Difco) (1% agar) at a final concentration of 50 μ l (ml medium)⁻¹. Under these experimental conditions, the penicillin N present in the assay does not form inhibition zones (Cortés *et al.*, 1984). The same reaction mixture was used to determine DAOC hydroxylase except that the deacetylcephalosporin C produced was determined by HPLC. Specific activities are given as pkat (pmol product formed s⁻¹) (g protein)⁻¹.

Separation and quantification of deacetoxycephalosporin C and deacetylcephalosporin C. The antibiotic products which originated from the reactions were analysed by HPLC through a μ Bondapak C18 (Waters) column (30 $\times$ 4 mm) equilibrated with 10 mM-acetate buffer pH 4.7-acetonitrile (99:1, v/v). The products were eluted by increasing linearly the flow, from 0.4 ml min⁻¹ (time zero) to 2 ml min⁻¹ at 3.5 min and to 3 ml min⁻¹ at 5.5 min. Under these conditions pure deacetoxycephalosporin C showed a retention time of 5.1 min and pure deacetylcephalosporin C eluted at 3.3 min.

Purification of DAOC synthase. The nucleic acids and nucleoproteins in the crude extract were precipitated by slowly adding a 1% (w/v) solution of protamine sulphate in TPD buffer to the S100 extract (1482 mg) at 4 °C, to a final concentration of 0.1%. The suspension was kept at 4 °C for 15 min and then centrifuged at 20000 g for 15 min. The supernate was mixed slowly with solid ammonium sulphate (enzyme grade) by stirring. All DAOC synthase activity precipitated in the 50–70% saturation ammonium sulphate fraction. This precipitate was dissolved in 120 ml TPD buffer and applied to a DEAE Sephacel column (30 × 1.5 cm) equilibrated with 300 mM-Tris/HCl, pH 9.5. The DAOC synthase activity was eluted with 300 mM-Tris/HCl in a linear pH gradient (9.5–6.5). The active fractions were pooled and concentrated 4-fold in an Amicon ultrafiltration cell with a PM-10 membrane. The concentrated preparation (11.2 mg in 5 ml) was applied to a Sephadex G-75 column (70 × 2.6 cm, Pharmacia) equilibrated with TPD buffer and the enzyme eluted with a constant flow of 8 ml h⁻¹. The whole purification procedure was done at 4 °C. Protein was detected with an UV detector (Pharmacia) set at 0.2 and a REC-1 recorder (Pharmacia). The molecular mass of the purified enzyme was determined by gel filtration through Sephadex G-75 column (70 × 2.6 cm) previously calibrated with the following standard proteins: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and bovine serum albumin (66 kDa).

SDS-PAGE. This was done as described by Laemmli (1970) in a BioRad Protean 16 cm vertical slab cell with circulating water at 4 °C for refrigeration, using 10% (w/v) polyacrylamide. Each track was loaded with 15–50 µg protein. The gels were stained with Coomassie Brilliant Blue R-250 or with silver nitrate. A set of molecular mass standards was used as controls.

Substrates and materials. DEAE Sephacel, Sephadex G-75 and G-100 (fine) were from Pharmacia. Penicillin N (95% purity) was kindly donated by H. H. Peter (Ciba Geigy, Basel, Switzerland). Deacetoxycephalosporin C and deacetylcephalosporin C were a gift of F. Salto (Antibióticos, León, Spain). Isopenicillin N (62% purity) was provided by P. van Dijk (Gist-Brocades, Delft, The Netherlands). The oxoacids and 2-oxoglutarate analogues tested were obtained from Sigma. All other chemicals were of reagent quality.

RESULTS

Cofactor requirements for optimum enzyme activity. The requirements of the DAOC synthase of *S. lactamdurans* were initially studied using a partially purified enzyme [160 pkat (g protein)⁻¹] in comparison with the cofactors already described for the analogous enzymes of *S. clavuligerus* (Jensen *et al.*, 1982) and *A. chrysogenum* (Hook *et al.*, 1979). When different cofactors or substrates were omitted from the reaction mixture (Table 1) the results indicated that only penicillin N, 2-oxoglutarate and Fe²⁺ ions were required for activity. Assays done under an atmosphere of nitrogen indicated that oxygen was also required. Ascorbate (a 2-electron donor), KCl, MgSO₄ and ATP were not necessary for activity, contrasting with the requirements of the expandase of *A. chrysogenum*. No variation in enzyme activity was found when the order of addition of the cofactors was altered. The reaction rate was linear for at least 3 h.

Optimum temperature and protein concentration. The DAOC synthase activity increased linearly with protein concentration in the range 0–0.75 mg protein (ml assay mixture)⁻¹. The optimum temperature for DAOC synthase activity was 25–30 °C; about 90% of the activity remained at 20 or 35 °C, but the activity decreased rapidly at temperatures above 35 °C or below 20 °C; 15% of the activity remained at either 5 or 40 °C. The stability of the enzyme at increasing temperature was measured by preincubating the enzyme for 60 min at different temperatures and then assaying the activity at 25 °C. The enzyme was stable up to 20–25 °C but increasing the temperature to 30 °C led to a 25% decrease in activity. The stability decreased linearly when the preincubation temperature was increased from 30 to 60 °C and no activity was observed after preincubation at 65 °C.

Optimum pH and pH stability. The DAOC synthase had a very broad pH optimum. Essentially the same activity was found when the buffers (50 mM-MOPS/NaOH in the pH range 5.0–7.0 and 50 mM-Tris/HCl in the pH range 7.0–11.0) were adjusted from pH 5.0 to pH 11.0. However, the stability of the enzyme in 50 mM-Tris/HCl changed at the different pH values tested. Maximum stability was at pH 9.0, with only 35% inactivation after 120 h at 4 °C, while the percentage inactivation at pH 8.0 and 7.0 was 70 and 80% respectively. The highest stability was observed when the enzyme was kept in 25 mM-Tris/HCl, pH 9.0. When 25 mM-MOPS/NaOH or HEPES/KOH buffer at the same pH (9.0) were used, activity was completely lost after only 72 h. Phosphate buffers were not used since the enzyme activity is inhibited by phosphate (Cortés *et al.*, 1986). Increasing the ionic strength of the Tris/HCl buffer to 100 mM decreased the stability of the enzyme, reducing activity by 50% in 48 h.

Table 1. *Substrate and cofactor requirements for DOAC synthase activity of S. lactamdurans*

The assay mixture contained, in a final volume of 0.4 ml: ascorbate (2.8 mM); FeSO_4 (0.045 mM); 2-oxoglutarate (1 mM); ATP (0.5 mM); KCl (7.5 mM); MgSO_4 (7.5 mM); penicillin N (0.2 mM); enzyme extract (0.4 mg protein in 0.3 ml); and Tris/HCl buffer (50 mM, pH 7.0).

Substrate omitted	Specific activity [pkat (g protein) ⁻¹]
None	160
Ascorbic acid	160
FeSO_4	0
2-Oxoglutarate	0
ATP	160
KCl	160
MgSO_4	160
Penicillin N	0

Identification of the reaction product. The product of the reaction was active against *E. coli* but not against *Micrococcus luteus* and was insensitive to penicillin-specific β -lactamases, i.e. it showed the characteristics of a cephalosporin. When the product of the reaction (using a partially purified enzyme preparation obtained after Sephadex G-75 filtration) was analysed by reverse phase HPLC a new peak was found which was not present at the beginning of the reaction. This peak eluted with a retention time of 5.1 min, and co-chromatographed with pure deacetoxycephalosporin C (Fig. 2). No peak corresponding to deacetylcephalosporin C (retention time 3.3 min) was found even after 120 min incubation, suggesting that the DAOC synthase activity was free of DAOC hydroxylase activity.

Purification of DAOC synthase. The steps followed in the purification of the enzyme are summarized in Table 2. All the DAOC synthase activity was recovered after treatment with protamine sulphate (0.1%), with only a slight increase in purity. Fractionation with ammonium sulphate (50–70% saturation) of the protamine sulphate supernate increased the enzyme purity 2-fold, with a recovery of 72%.

After this step several other purification procedures including dialysis, Sephadex G-25 gel filtration or ion exchange chromatography through DEAE Sephacel followed by elution with a concentration gradient of Tris/HCl, pH 7.0 (25–300 mM) were all unsuccessful leading to total loss of activity. Ion exchange chromatography in DEAE-HPLC also resulted in a considerable loss (about 95%) of activity. These results probably reflect the enzyme instability discussed above.

Good purification was possible, however, by anion exchange chromatography in DEAE-Sephacel followed by elution with a pH gradient of 300 mM-Tris/HCl in the pH range 6.5–9.5. Advantage was taken of the broad pH range of activity and stability of the enzyme at pH 9.0. As shown in Fig. 3, the enzyme eluted when the gradient reached a pH of 7.5. Under these conditions a 20-fold purification was obtained with a 15% recovery (Table 2). Although the recovery of activity was low, this step removed many of the contaminant proteins as judged by PAGE (see below). The Amicon-concentrated active samples (11.2 mg protein) were subsequently applied to a Sephadex G-75 column (Fig. 4), to give a final purification of 75.6-fold, with a recovery of 19% of the activity (i.e. with slight increase in the recovery of activity over the previous step of purification). This increase in the recovery suggests that in the eluate of the anion exchange column by pH gradient, the enzyme is partially inhibited either by some inhibitor which is removed during gel filtration or by a pH effect. The active samples (Fig. 4) were pooled and kept frozen at -70°C . Attempts to further purify the enzyme by DEAE-HPLC led to the loss of the enzyme activity.

Molecular mass. This was determined by gel filtration through a Sephadex G-75 column calibrated as described in Methods. The DAOC synthase eluted with a K_{av} of 0.484, corresponding to a molecular mass of 27000 Da $\pm$ 1000 (Fig. 4).

SDS-PAGE. The purity of enzyme samples after the purification steps was followed by SDS-PAGE (Fig. 5). The main purification step was anion exchange chromatography followed by

Fig. 2. HPLC analysis of the reaction product(s) of DAOC synthase. (a) Separation of a mixture of pure deacetylcephalosporin C (DAC) (retention time 3.3 min) and deacetoxycephalosporin C (DAOC) (retention time 5.1 min). (b) Reaction products. (c) A mixture of the reaction products with the pure antibiotics used in (a). The peak of deacetoxycephalosporin C is indicated by an arrow.

Table 2. Partial purification of DAOC synthase of *S. lactamdurans*

Purification step	Activity (pkat)	Protein (mg)	Specific activity [pkat (g protein) ⁻¹]	Recovery of activity (%)	Purification (-fold)
Crude extract	119	1482	80.2	100	1.0
Protamine sulphate	117	1364	85.7	98	1.1
Ammonium sulphate (50–70%) precipitate	86	510	168.0	72	2.0
Anion exchange–pH gradient	18	11.2	1607.1	15	20.0
Sephadex G-75 gel filtration	23	3.8	6052.6	19	75.6

elution with a pH gradient (Fig. 5, lane E). Several of the contaminant proteins were removed during the subsequent gel filtration through Sephadex G-75 (Fig. 5, lane F). The DAOC synthase appeared as an intense band with minor contaminant proteins after Sephadex G-75 filtration (Fig. 5, lane F). The molecular mass estimated by PAGE was 28 000 Da which agrees with the molecular mass established by gel filtration.

Kinetics of the DAOC synthase. The kinetics of DAOC synthase for the substrate and cofactors of the reaction were determined using enzyme of the highest purity (1600 pkat g⁻¹). In one experiment the concentration of 2-oxoglutarate (1–50 µM) was varied while the other two

Fig. 3. Anion exchange chromatography of DAOC synthase in DEAE Sephacel. The enzyme was eluted using a pH gradient. ●, DAOC synthase activity; —, A_{280} (protein); ---, pH gradient (9.5 to 6.5) (300 mM-Tris/HCl buffer).

Fig. 4. Gel filtration of DAOC synthase through Sephadex G-75. (a) Purification of concentrated extract. ●, DAOC synthase activity; —, A_{280} (protein). (b) Determination of molecular mass in comparison with protein standards. The DAOC synthase had a molecular mass of 27000 Da $\pm$ 1000.

substrates were kept at the concentrations indicated in Methods. In another experiment, the concentration of penicillin N (0.01–0.15 mM) or Fe^{2+} (0.045–1 mM) were varied while the other parameters were kept constant. The K_m and $V_{\max}$ in every case were determined from Lineweaver–Burk double reciprocal plots. For penicillin N, the apparent K_m was 52 μM and the

Fig. 5. Electrophoresis in SDS-PAGE of samples of DAOC synthase after different steps of purification. Lanes A and G, molecular mass markers. Lane B, S100 cell-free extracts; lane C, protamine sulphate supernate; lane D, ammonium sulphate (50–70%) precipitate; lane E, active fractions eluted from DEAE Sephacel using a pH gradient; lane F, active fraction obtained after Sephadex G-75 gel filtration. The DAOC synthase band is indicated by an arrow. About 50 μg protein were loaded in tracks A to C; tracks E and F were loaded with 15 μg protein.

V_{max} was 7.6 pmol DAOC formed $(\text{ml} \cdot \text{s})^{-1}$. The apparent K_m for 2-oxoglutarate was 3 μM with a V_{max} of 6.6 pmol $(\text{ml} \cdot \text{s})^{-1}$. Finally, for the cofactor Fe^{2+} , the apparent K_m was 71 μM with a V_{max} of 7.1 pmol $(\text{ml} \cdot \text{s})^{-1}$.

Substrate specificity. Enzyme preparations from the last purification step (Sephadex G-75 filtration) were used to study the substrate and cofactor requirements of the DAOC synthase. Isopenicillin N, penicillin G and 6-aminopenicillanic acid (all 1 mM) were tested either as substrates of the expandase (instead of the natural substrate penicillin N) or as competitive inhibitors of penicillin N. None of these β -lactams were substrates of the DAOC synthase, indicating a high substrate specificity. Moreover, none of these compounds inhibited the formation of DAOC from penicillin N.

Effect of 2-oxoglutarate analogues and of cations. Several amino acids and oxoacids were tested in order to see if they could either serve as cofactors or compete with 2-oxoglutarate. Neither glutamate, succinate, 2-oxoisovalerate, 2-oxocaproate nor oxalacetate had any effect on the reaction, but 2-oxobutyrate completely inhibited the formation of deacetoxycephalosporin C and 3-oxoadipate produced a 56% inhibition (Table 3). However, these two analogues could not be used as cofactor in the reaction since no DAOC was formed when 2-oxobutyrate or 3-oxoadipate were used instead of 2-oxoglutarate.

K^+ , Na^+ , Hg^{2+} or Fe^{3+} (all 1 mM) did not affect enzyme activity but Co^{2+} , Zn^{2+} and Cu^{2+} strongly inhibited the formation of DAOC (79, 84 and 100% respectively). Fe^{3+} did not inhibit enzyme activity and it could be used instead of Fe^{2+} under the reducing conditions existing in the reaction mixture.

Table 3. *Effect of cations and 2-oxoglutarate analogues on DAOC synthase activity*

The assays were done using a purified DAOC synthase preparation obtained after Sephadex G-75 filtration. In this particular experiment, the control reaction mixture contained 2-oxoglutarate (0.05 mM) and Fe^{2+} (225 μM). No activity was obtained when 2-oxoglutarate in the reaction mixture was replaced by any of the 2-oxoglutarate analogues or when Fe^{2+} was replaced by any of the other ions indicated in the table (except Fe^{3+}).

Addition	Specific activity [pkat (g protein) ⁻¹]	Inhibition (%)
None (control)	970	0
Glutamate (0.1 mM)	970	0
Succinate (0.1 mM)	970	0
2-Oxoisovalerate (0.1 mM)	970	0
2-Oxocaproate (0.1 mM)	970	0
2-Oxobutyrate (0.1 mM)	0	100
Oxalacetate (0.1 mM)	970	0
3-Oxoadipate (0.1 mM)	420	56
Fe^{3+} (1 mM)	970	0
Hg^{2+} (1 mM)	970	0
Co^{2+} (1 mM)	210	79
Cu^{2+} (1 mM)	0	100
Zn^{2+} (1 mM)	140	84
K^{+} (1 mM)	970	0
Na^{+} (1 mM)	970	0

DISCUSSION

S. lactamdurans and other micro-organisms that synthesize cephalosporins and cephamycins do so by oxidative ring expansion of penicillin N to deacetoxycephalosporin C. Penicillin N itself is formed in this micro-organism by cyclization of the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (Castro *et al.*, 1988) followed by an L to D epimerization of the α -aminoadipyl moiety during the conversion of isopenicillin N to penicillin N (Castro *et al.*, 1985).

Because of the instability of the DAOC synthases of *A. chrysogenum* (Yoshida *et al.*, 1978) and *S. clavuligerus* (Jensen *et al.*, 1982) and the inherent difficulty in purifying them (Kupka *et al.*, 1983a, b; Jensen *et al.*, 1985) some uncertainty concerning the necessary cofactors has remained. The DAOC synthase of *S. lactamdurans* requires oxygen and 2-oxoglutarate and is an intermolecular dioxygenase functionally similar to the enzymes of *A. chrysogenum* and *S. clavuligerus*, which suggests a common evolutionary origin of these enzymes in the different producers of cephalosporin and cephamycin. However, there are some differences in the cofactor requirements and in the molecular mass. The DAOC synthase of *S. lactamdurans* requires oxygen, a thiol-containing reducing agent (DTT), Fe^{2+} and 2-oxoglutarate as cofactors but in contrast to the enzyme of *A. chrysogenum* (Hook *et al.*, 1979; Kupka *et al.*, 1983a, b; Felix *et al.*, 1981) it does not require ATP, ascorbic acid, or Mg^{2+} or K^{+} ions. Therefore, it should be named a synthase rather than synthetase. The DAOC synthase of *S. lactamdurans* shows more similarity to the enzyme of *S. clavuligerus* that also showed an absolute requirement for 2-oxoglutarate but does not need ATP (Jensen *et al.*, 1982).

The apparent K_m for 2-oxoglutarate of the DAOC synthase of *S. lactamdurans* was 3 μM which is an order of magnitude less (i.e. the enzyme shows more affinity) than the K_m value of the enzyme of *A. chrysogenum* (40 μM). The absolute requirement for 2-oxoglutarate of the DAOC synthase of *S. lactamdurans*, which has also been shown unequivocally in the fungal enzyme (Kupka *et al.*, 1983a) in contrast to previous claims (Hook *et al.*, 1979), indicates that the expandases characterized so far are intermolecular dioxygenases which use 2-oxoglutarate as substrate (Abbot & Underfriend, 1974). The requirement for 2-oxoglutarate of the DAOC synthase of *S. lactamdurans* was rather specific since neither glutamate, succinate, oxalacetate, 2-oxobutyrate, 2-oxoisovalerate, 2-oxocaproate or 3-oxoadipate were used as cofactors. 2-Oxobutyrate completely inhibited enzyme activity and 3-oxoadipate produced a 56% reduction, probably as a result of a competitive inhibition. For the fungal expandase, 2-oxoadipate was an

alternative cofactor with about 30% of the activity of 2-oxoglutarate. This would suggest that the fungal enzyme has a broader cofactor specificity. However, the authors pointed out that there was a possibility that contaminant transaminases provide 2-oxoglutarate (from glutamate) since the study was done with a partially purified enzyme (Kupka *et al.*, 1983a).

Ascorbic acid stimulates the fungal DAOC synthase (Hook *et al.*, 1979) and the same apparently occurs in *S. clavuligerus* (Jensen *et al.*, 1982). The *S. lactamdurans* enzyme is different in that it lacks the requirement for ascorbic acid, a reducing cofactor of standard dioxygenases (Myllylä *et al.*, 1978). However, it is important to keep in mind that the enzyme was always kept in DTT to preserve activity.

In *S. lactamdurans* the expandase reaction proceeds independently of the order in which the substrate and cofactors are added to the reaction mixture, in contrast to the behaviour described for the DAOC synthase of *A. chrysogenum* (Shen *et al.*, 1984).

The DAOC synthase of *S. lactamdurans* showed a high specificity for the nature of the side chain in the penicillin substrate. Only penicillin N, but not isopenicillin N, penicillin G or 6-aminopenicillanic acid served as substrates. The expandases of *A. chrysogenum* (Kupka *et al.*, 1983a) and *S. clavuligerus* (Jensen *et al.*, 1982) showed the same specificity. However, unlike the fungal enzyme, the DAOC synthase of *S. lactamdurans* was not inhibited by penicillin G.

The ring expansion activity is released from the mycelium by sonication (i.e. is not associated with the membrane systems) as occurs also with the expandases of *A. chrysogenum* and *S. clavuligerus* (Kupka *et al.*, 1983a; Jensen *et al.*, 1982). The enzyme of *S. lactamdurans* has a molecular mass of 27 kDa slightly lower than that of the enzyme of *A. chrysogenum* (31 kDa) (Scheidegger *et al.*, 1984) and *S. clavuligerus* (29.5 kDa) (Jensen *et al.*, 1985). It appears to be a monomer since the molecular mass of the natural (non-denatured) form, determined by gel filtration is identical to that of the SDS-denatured protein as estimated by SDS-PAGE.

Despite the instability characteristic of all expandases, the DAOC synthase of *S. lactamdurans* was purified to near homogeneity by a combination of chromatographic techniques, thanks to the development of an ion exchange procedure based on a pH gradient.

The purified enzyme showed no DAOC hydroxylase activity, which is, however, present in cephamycin-synthesizing cells, suggesting that the two activities are separated in the chromatographic procedures and therefore are probably two separate enzymes as in *S. clavuligerus* (Jensen *et al.*, 1985), in contrast to *A. chrysogenum*, where they appear to be located in a single protein (Scheidegger *et al.*, 1984). In summary, the expandase of *S. lactamdurans* is more closely related to the enzyme of *S. clavuligerus* than to the enzyme of *A. chrysogenum*, which suggests that even if they have a common evolutionary origin the fungal enzyme diverged differently from the bacterial (streptomycete) DAOC synthases.

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